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Phytohemagglutinin binds to the 20-kDa molecule of the T3 complex*

Current findings have suggested that the T3 molecular complex is an essential antigenic signal transducer during T cell activation. Lectins, such as phytohemagglutinin (PHA), activate T cells nonspecifically. Concesivably, lectins may mediate their stimulatory action by affecting the T3 complex. In the present investigation we have studied the involvement of the T3 molecular complex in the PHA-mediated activation of T cells. We selectively modulated the surface expression of T3 molecules by anti-T3 antibody and subsequently tested the ability of the modulated cells to respond to PHA. Reduction of T3 expression by 70% resulted in 80% inhibition of the PHA response. This effect was specific for T3 since modulation of other T cell surface molecules (T4, T8) did not affect the PHA-induced mitogenesis. To determine if PHA could interact directly with the T3 complex, immunoblotting (Western blot) analyses of anti-T3 immunoprecipitates were performed. A 20-kDa member of the T3 complex reacted not only with the anti-T3 antibody, but also with PHA itself. These results provide the first evidence for direct binding of PHA to one of the molecules of the T3 complex. The combined data suggest that a major pathway of PHA-induced T cell activation involves the T3 complex. Possible activation mechanisms are discussed.

1 Introduction

Recent work has focused attention on the T3 complex as the probable transducer of antigenic signals in T cell activation [1–3]. This complex is found on almost all T cells and has been associated with many T cell functions (reviewed in [1]). The T3 complex consists of 3 transmembrane polypeptides, two of which are N-glycosylated and one of which is believed to be unglycosylated [4–10]. The T3 complex is variably associated with the antigen recognition heterodimer, which may associate with the T3 complex both in co-capping [11] and in immunoprecipitation experiments [10, 12]. Several monoclonal antibodies (mAb) with mitogenic activity are known to bind to at least one of the members of the complex, most likely to the 20-kDa polypeptide that has both mannose and galactose residues [4, 10]. As galactose residues are the targets specified by the plant mitogen phytohemagglutinin (PHA) [13], it seemed possible that PHA could interact with the 20-kDa molecule. Recent reports have indeed indicated that the presence of the T3 complex is required for T cell response to mitogens [14–16]. Direct evidence of physical interaction of PHA with the molecules of the T3 complex is required for T cell response to mitogens [14–16]. Direct evidence of physical interaction of PHA with the molecules of the T3 complex, however, was still lacking. In the present report we investigated this question by immunoblot analysis of the molecules precipitated by anti-T3 antibodies. The results of these studies demonstrated that the anti-T3-reactive 20-kDa protein can interact with PHA and reiterate the required presence of the T3 complex for mitogen-induced cell activation.

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2 Materials and methods

2.1 Cell lines

The human malignant T lymphoblastoid cells HPB [17] and Molt-3 [18] and several Epstein-Barr virus-transformed B cell lines were maintained in logarithmic growth in RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 5% fetal bovine serum.

2.2 Reagents

The mouse monoclonal antibodies (mAb) 64.1 (New England Nuclear, Cambridge, MA), and OKT3, OKT4 and OKT8 (Ortho Pharmaceutical Corp., Raritan, NJ) were used for immunofluorescence, functional studies and immunoprecipitation. Highly purified PHA-L was obtained from E. Y. Labs (San Mateo, CA). This reagent shows a single band on electrophoresis, has mitogenic activity for lymphocytes, but no agglutinating activity with erythrocytes (E. Y. Labs technical bulletin). Phorbol myristate acetate (PMA, Sigma Chemical Co., St. Louis, MO) was made as a stock solution in ethyl alcohol and stored at –20 °C until use.

2.3 Preparation of purified T cells

Peripheral blood mononuclear cells were obtained from heparinized blood by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient centrifugation [19]. T cells were prepared by two cycles of rosetting with neuraminidase-treated sheep red cells [20]. The erythrocytes were removed by hypotonic lysis in Tris-ammonium chloride and contaminating monocytes removed by two cycles of iron carbonyl treatment [21]. The resulting cells stained > 99% with mAb Leu-5 that recognizes

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Abbreviations: PHA: Phytohemagglutinin PMA: Phorbol myristate acetate FACS: Fluorescence-activated cell sorter mAb: Monoclonal antibody(ies)

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the sheep red blood cell receptor (Becton Dickinson, Sunnyvale, CA) [22]. These preparations were less than 1% esterase positive [23] and displayed no proliferative response when cultured with the mitogenic antibody 64.1 alone.

2.4 Modulation of surface molecules

Purified T cells at 4×10^6/ml were incubated for 1 h at 4°C with mAb 64.1 or OKT3 (100 ng/ml) or a mixture of mAb OKT4 and OKT8 (total concentration 100 ng/ml) or medium. After washing, a goat anti-mouse IgG antibody (1:10) was added and incubation continued for an additional hour at 4°C. The cells were washed and incubated overnight at 37°C in RPMI 1640 supplemented as above, with 5% autologous plasma substituting for fetal bovine serum. The cycle of antibody incubations was repeated with a 4-h incubation at 37°C. A portion of the cells was subjected to immunofluorescence analysis while the remaining cells were activated with PHA (see below).

2.5 Immunofluorescence analysis

To assess residual T3 molecules after modulation, the cells were stained directly with fluorescein-conjugated OKT3 (1:100) or indirectly with OKT3 (1:100) followed by fluoresceinated goat anti-mouse IgG (1/10). Both techniques gave comparable results. Specificity controls modulated by OKT4 and OKT8 were stained with OKT4 (1/100) and fluorescein-labeled goat anti-mouse IgG. The above staining procedure has been previously described [24]. Cytofluorographic analysis was performed with a fluorescence-activated cell sorter (FACS II; Becton Dickinson Electronic Laboratories, Mountain View, CA) [25]. Both percent fluorescent cells and the mean fluorescence intensity of the positive cells were determined after counting 10000 cells per sample.

2.6 Proliferation assays

Samples of modulated T cells or controls were incubated in serum-free medium containing 2% PHA with 50 ng/ml PMA for 15 min at 37°C, followed by 45 min at room temperature. After washing, 2×10^5 viable cells were incubated for 3 days at 37°C in triplicate microtiter tray wells (Costar, Cambridge, MA; #3596) in medium supplemented with 2% autologous plasma and 50 ng/ml PMA. Proliferation was assessed following a 4-h pulse with [^{3}H]thymidine (1 μCi/well = 37 kBq/well). Results are expressed as average counts per minute (cpm).

2.7 Immunoprecipitation

Cell lysates were prepared using 1% Nonidet-P40 in 0.4% sodium dodecyl sulfate and 1 mM phenylmethylsulfonyl fluoride. After centrifugation at $10000 \times g$ for 5 min, the lysates were depleted of nonspecific binding components by incubation with formalin-fixed *Staphylococcus aureus*, Cowan strain I (SAC). The bacterial cells were removed at $10000 \times g$ (2 min) and the supernatants were incubated with: (a) a mixture (1:1) of 64.1 and OKT3 at final dilutions of 1:10, or (b) a mixture (1:1) of OKT4 and OKT8, both at a final concentration of 1:10. The combination of the two anti-T3-reactive antibodies was used to increase the efficiency of precipitation, as they do not cross-block each other's binding in immunofluorescence assays (C. Tsoukas, unpublished). After a 4-h incubation at room temperature, a 20% suspension of SAC was added and the mixtures were incubated overnight at 4°C with mixing. Following incubation, the bacteria were centrifuged ($10000 \times g$; 1 min) and washed three times in phosphate-buffered saline. Complexes were then eluted from the bacteria by boiling 5 min in electrophoresis sample buffer prepared as described by Laemmli [26].

2.8 Gel electrophoresis and immunoblotting analysis

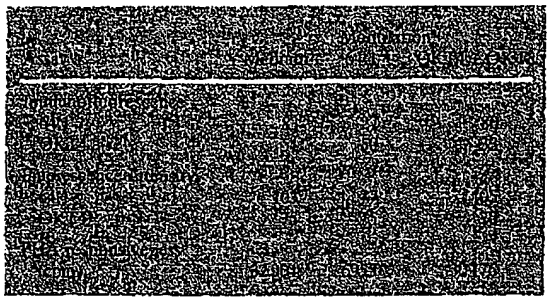
Eluates were cleared by centrifugation ($10000 \times g$) and electrophoresed in a 10% polyacrylamide-sodium dodecyl sulfate slab gel (10 cm length) using a 5% loading gel, as described [26]. Gels were electroblotted onto nitrocellulose (NC) paper. The NC paper was cut in sections for incubation with either mAb 64.1 (1:200), or with peroxidase-labeled PHA. Strips incubated with the mouse mAb 64.1 (1 h) were quenched by overnight incubation with 1:100 normal human serum in 2% powdered milk phosphate-buffered saline (PBS), washed and then incubated with a peroxidase-labeled goat anti-mouse IgG (1:200, 1 h). Quenching of the strips designated for PHA was performed using 5% bovine serum albumin (BSA) overnight. These strips were then incubated with a 1:150 dilution of peroxidase-labeled PHA for 1¼ h and washed for 1 h with several changes of 5% BSA. Both sets of strips were washed two times (10 min each) in PBS and the color was developed.

3 Results and discussion

Treatment with anti-T3 mAb caused a selective reduction of T3 molecule(s) on the surface of purified peripheral T cells and a parallel decrease in PHA responsiveness. Table 1 displays representative data from one of three replicate experiments. Modulation by mAb 64.1 resulted in 67% reduction in $T3^+$ cells and a 25% reduction fluorescence intensity (Table 1). These modulated cells displayed a 79% inhibition of PHA-induced proliferation (Table 1). This phenomenon was specific for the anti-T3 antibody, since modulation with other mAb (OKT4 and OKT8) did not alter PHA responsiveness. This suggests that the amount of surface T3 is a critical variable in PHA activation. Extension of this observation should prove helpful for understanding the threshold requirements of membrane signalling that result in T cell activation. Further, these observations exclude the T4 and T8 molecules as functionally associated with the T3 complex.

Other investigators have shown that modulation of the T3 molecule can decrease the response of T cells to mitogens [16]. Our immunofluorescence data extend these findings to indicate that the effect of modulation on PHA responsiveness is specific for the molecules that comprise the T3 complex. Neither our data, the previously published functional correlates, nor the recently described PHA-mediated cross-blocking of anti-T3 antibody reactivity with lymphocytes [27] provide unequivocal evidence for direct binding of PHA to the T3 complex. To address this issue, we immunoprecipitated the T3 complex of two tumor T cell lines, Molt-3 and HPB, and subsequently analyzed the precipitates by immunoblotting using either anti-T3 antibody or PHA. Preliminary controls established that all four polypeptide species associated with the T3

Table 1. Modulation of surface T3 complex decreases the proliferative response of purified T cells to PHA



a) PUrified peripheral T cells were modulated with medium or mAb 64.1, or a mixture (1 : 1) of mAb OKT4 and OKT8 as described in Sect. 2.4. All data are from one representative experiment.
b) Following modulation, cells were stained with 64.1 and a fluoresceinated anti-mouse antibody, and then were analyzed in the FACS.
c) Median fluorescence intensity of positive cells as established by FACS analysis.
d) An aliquot of cells was incubated with 2% PHA and 50 ng/ml PMA for 1 h, washed and then cultured for three days. Responsiveness was measured by [^{3}H]thymidine uptake (see Sect. 2.6).

complex were present in these preparations on both gels and blots (Fig. 1). Fig. 2 clearly shows a 20-kDa band identified by the anti-T3 antibody 64.1 (lane 1) that migrates identically as the 20-kDa band from the two T cell lines recognized by PHA (lanes 2 and 3). Importantly, this precipitated 20-kDa species was never found in B cell precipitates (lanes 6 and 7), nor was it seen in precipitates made from the unrelated antibodies OKT4 and OKT8 (lanes 4 and 5). The T3 molecules on the tumor T cell line HPB are identical to those of peripheral T cells as determined by gel electrophoresis and peptide map analyses [28]. Antibody 64.1 recognizes 83% of Molt-3 and 87% of HPB cells by indirect immunofluorescence. Both the T4 and T8 molecules were precipitated in HPB lysates.

PHA also bound a 42-kDa molecule in both T and B cell precipitates. The characteristics of this band were consistent with actin or protein A, as judged by molecular weight and reactivity with an anti-actin antibody. B cell lysates also had a higher molecular weight band recognized by PHA whose molecular weight is consistent with the heavy chain of IgG. The binding of PHA to B cells has been established [29].

An unresolved issue remains whether anti-T3 antibodies recognize a determinant common to all the members of the T3 complex, or rather bind to only one of the components, and co-precipitate associated molecules. The data in Fig. 2 indicate that under our experimental conditions mAb 64.1 reacts primarily with the 20-kDa component of the complex. This supports the notion that the T3 reactive antibodies may indeed bind preferentially to one component of the T3 complex. This suggests that: (a) the three members of the T3 complex and possibly the antigen-specific heterodimer interact strongly enough (via actin?) to survive precipitation procedures; (b) the 20-kDa protein may be present in greater amounts than the other parts of the T3 complex, or (c) the epitope recognized by 64.1 is more stably expressed under these conditions. Excluding the last possibility, immunoprecipitation defines the 20-kDa species as the molecule with which the antibody would have the greatest interaction.

As noted, the T3 molecular complex is noncovalently associated on the T cell surface with the antigen receptor heterodimer [12]. Since the receptor proteins have glycosylation sites, the possibility exists that PHA or other lectins may mediate their mitogenic effects by binding to both T3 and receptor molecules. This interpretation is consistent with

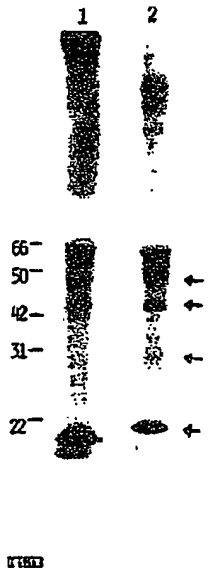


Figure 1. Immunoprecipitates of the T3 complex are present on both gels and blots. HPB cells were surface labeled with ^{125}I using the chloramine-T method (Iodobeads, Pierce Chemical Co., Rockford, IL). Cells were washed, lysed and immunoprecipitated using OKT3 and nonstringent conditions, as described in Sect. 2.7. Comparison of surface-labeled material on gels (lane 1) and blots (lane 2) showed the characteristic polypeptides (20, 28, 44 and 50 kDa) to be present (arrows). B cell controls appear on top.

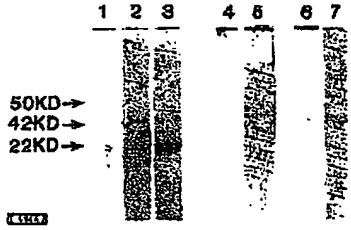


Figure 2. PHA recognizes the 20-kDa molecules immunoprecipitated by antibodies against the T3 complex. Lysates from the tumor T cell lines Molt-3 and HPB or from B cell lines were precipitated with mAb, and the immunoprecipitates were electrophoresed and then analyzed by immunoblotting. The lanes are identified as follows

Lane	Cell lysate	Precipitating reagent	Developing reagent
1	Molt-3	OKT3 + 64.1	64.1
2	Molt-3	OKT3 + 64.1	PHA
3	HPB	OKT3 + 64.1	PHA
4	Molt-3	OKT4 + OKT8	64.1
5	Molt-3	OKT4 + OKT8	PHA
6	B cells	OKT3 + 64.1	64.1
7	B cells	OKT3 + 64.1	PHA

recent data by Weiss and Stobo [30] that describes mutants with coordinant decrease or absence of both receptor and T3 molecules. Conceivably, activation would then result when cross-linking of the T3 and receptor complex by the multivalent lectin established a necessary propinquity. Broadly reactive antibodies to the antigen receptor on T cells will provide the needed probe to answer this question.

These findings established the direct binding of PHA to the 20-kDa T3 polypeptide in T3 precipitates. They do not directly show that the binding may occur to the complex as it is seen on the T cell, nor do they require, if PHA does stimulate throught the T3 complex, that this complex is the only mechanism by which T cells may be activated. Considering, however: (a) when the T3 complex is decreased or absent, mitogen responses are concurrently decreased or absent, (b) the ability of monoclonal antibodies specifically directed against the T3 complex to activate T cells, (c) the reported cross-blocking of antibodies against the T3 complex by PHA as seen by immunofluorescence [27], and (d) the ability of the mitogen to bind to the 20-kDa T3 molecule reported here, a role for the T3 complex in T cell activation by mitogens is inescapable. Our data can be offered in the light of three alternate activation mechanisms. First, that indeed PHA may activate T cells by direct interaction with the T3 molecule. Second, the binding of mitogen to numerous sites on the T cell surface may effect sufficient membrane perturbation to induce a calcium influx [15] and generation of diacylglycerols. This, in turn, could signal the T3 complex and effect activation. Third, the mitogen may bind to other activation molecules (T11?) having functional linkage with the T3 complex. We suggest that none of the above mechanisms need be exclusive.

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